REMARKS

Claims 1-12 are presently pending and under consideration. Claim 7 and previously withdrawn claims 13-18 have been canceled. Claim 1 has been amended herein. The amendments are supported throughout the specification, for example, by claim 7 as originally filed. No new matter has been introduced by way of these amendments and entry is respectfully requested. Upon entry of the amendments, claims 1-6 and 8-12 will be pending and under consideration.

Regarding 35 U.S.C. § 102

The rejection of claim 1 under 35 U.S.C. §102(a) as allegedly anticipated by Fitzsimons et al., Gene Therapy, 8:1675-1681 (2001) is respectfully traversed. The Office alleges that Fitzsimmons et al. discloses the vector of Claim 1. Claim 1 has been amended to recite an adenoviral vector, thereby rendering moot the instant rejection. Contrary to the Examiner's assertion, Fitzsimons discloses a recombinant adeno-associated virus (rAAV) viral vector, but not an adenoviral vector as claimed in amended claim 1. Since Fitzsimons does not teach every element of claim 1, this reference cannot support an anticipation rejection of claim 1. Accordingly, removal of the rejection of claim 1 under 35 U.S.C. §102(a) as allegedly anticipated by Fitzsimons et al., Gene Therapy, 8:1675-1681 (2001) is respectfully requested.

Regarding 35 U.S.C. § 103

Applicants respectfully traverse the rejection of claims 1-3, 5 and 7-12 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Fitzsimons et al., *Gene Therapy*, 8:1675-1681 (2001) in view of Nakagawa et al., *European Journal of Pharmaceutical Sciences* 13:53-60 (2001).

Applicant respectfully points out the following deficiencies with regard to the aforementioned rejection: (1) The references, viewed alone or in combination, do not teach or suggest all of the elements of the rejected claims, and (2) the claimed invention represents more than the predictable use of the elements described in the cited prior art as evidenced by the unexpected results described in detail below.

The examiner bears the burden of establishing a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3 1531, 1532, (Fed. Cir. 1993). Only if this burden is met does the burden of coming forward with rebuttal argument or evidence shift to the applicant. *Id.* at 1532. When the references cited by the examiner fail to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Fine*, 837 f.2d 1071, 1074 (Fed. Cir. 1988).

To support a *prima facie* finding of obviousness, it is required that the prior art references must teach or suggest all the claim limitations. *In re Royka*, 490 F.2d 981, 985 (CCPA 1974).

When the PTO shows *prima facie* obviousness, the burden then shifts to the applicant[s] to rebut." In re Mayne, 104 F.3d 1339, 1342, 41 USPQ2d 1451, 1454 (Fed. Cir. 1997). "Such rebuttal or argument can consist of a comparison of test data showing that the claimed compositions possess unexpectedly improved properties or properties that the prior art does not have..." In re Dillon, 919 F.2d 688, 692-93, 16 USPQ2d 1897, 1901 (Fed. Cir. 1990)(en banc).

A claimed invention is unobvious when it represents "more than the predictable use of prior art elements according to their established functions." *See KSR Int'l Co. v. Teleflex Inc., 550 U.S. __, 2007 WL 1237837, at *36 (2007).* As disclosed in the specification, the claimed invention is more than the predictable use of prior art elements according to their established functions.

<u>*Lack of Prima Facie Case*</u>

As amended, claim 1 is directed to a recombinant vector containing an insert having the general structure tTA-intron1-TK+-TetO7-CMV+-intron2-transgene, wherein the vector is an adenovirus. Fitzsimons describes a recombinant adeno-associated virus (rAAV) viral vector, but does not teach or suggest an adenoviral vector as claimed in amended claim 1. This deficiency is not cured by the secondary reference by Nagawa et al. To support a *prima facie* finding of obviousness, it is required that the prior art references must teach or suggest all the claim limitations. *In re Royka*, 490 F.2d 981, 985 (CCPA 1974). Applicants respectfully submit that this requirement for a *prima facie* case has not been met with regard to the instant rejection.

<u>*Unexpected Results*</u>

Expression of the construct described by Fitzsimons et al. was less than two-fold higher as compared to expression in a plasmid (see Figure 4). Suppression (repression) is shown

between 100-fold to 200-fold with a maximum at 204-fold (cf. Figures 5 and 7). Fitzsimons only discloses constructs expressing luciferase, whereas constructs containing IL12 or other transgenes are not disclosed at all.

This high efficiency of gene expression observed with the vectors of the present invention represents an unexpected result that amounts to much more than the predictable use of the prior art elements according to their established functions. In particular, gene expression for the claimed adenoviral vector construct is much higher than for the Fitzsimons et al. rAAV vector. For example, for a construct containing IL12 as transgene, up to 1,000 μg/24 h/106 cells could be expressed in a linear, m.o.i. dependent fashion for various cell lines using the same transgene (IL12) in 48 hours at a m.o.i. of 10 (expression of up to 200 μg). Attached as Exhibit 1 to this response is a paper co-authored by the inventor, Block et al., *Journal of Gene Medicine* 5:190-200 (2003), describing the unexpected results.

The high efficiency of gene expression was not only unexpected, but was confirmed in various cell lines and for various transgenes with a comparable order of magnitude. Furthermore, the claimed construct provides 16,000-fold suppression for expression of the luciferase gene and 6,000-fold for IL12 expression. The order of magnitude of suppression was also completely surprising and unexpected by the skilled artisan. In this context, attached as Exhibit 2 is Figure 1, which shows expression of hIL12 following infection of human colon carcinoma cells with 10 m.o.i. and incubation over 24 hours using various concentrations of doxycyclin using the claimed adenoviral vectors. Determination of IL12 in supernatant and cell lysate is shown. The data obtained for the claimed vectors according to claim 1 support the unexpected high native expression and suppressibility achieved with the claimed vectors and represent their application in clinical studies.

Utilizing the vectors of the invention, various animal experiments were performed with respect to toxicity and efficiency. Due to the unexpected high suppressibility of IL12 expression, complete protection in C57B16 mice could be achieved by adding doxycyclin (Dox) to the drinking water following systemic application of a 100 % lethal vector dosage. Further, attached as Exhibit 3, is Figure 2, which shows that intra-tumoral application led to highly significant tumor regression in all treated animals showing a long-term survival of 25%. Attached as Exhibit 4, is Figure 3, which shows tumors after day 18. Well observable are large tumors under control (mock) or when using control vector Ad.DL312, whereas no tumors or only very small

tumors were observed following application of vector Ad3r-IL12 of the invention.

For the Examiner's convenience, the significant differences of the vector according to the invention as compared to the expression system according to Fitzsimons is shown in the following Table:

	Fitzsimons et al.	**Vector of the invention**
Dox-regulated suppressibility (IL12)	Not tested	6,000-fold
Dox-regulated suppressibility (Luc)	< 204-fold	16,000-fold
IL12 expression (µg/24h/10^6 cells)	Not tested	1.000
Tumor regression	Not tested	Almost completely

Overall, the unexpectedly high expression as well as the surprising high doxycyclin-dependent suppressibility of the claimed vector constructs provide significant advantages regarding safety and efficiency when treating malignant diseases.

The skilled person familiar with the cited references would not have been motivated to specifically modify the Fitzsimons reference to select the adenoviral vector system of the current invention. Moreover, the skilled person would not have expected the significantly higher efficiency of gene expression and the higher suppression rates compared to the expression systems described in the cited references.

The publication of Nakagawa et al. does nothing to cure the deficiencies of Fitzsimons et al. since the reference merely describes a Tetracyclin-regulated expression system in which IL12 is inserted as transgene into a recombinant adenovirus vector. For the reasons described in detail above, the skilled person would not have expected the high gene expression and suppressibility associated with the vector constructs of the current invention. With respect to the Examiner's argument at page 7 of the Office Action that Fitzsimons et al. have minimized the size of the cassette and decreased basal leakiness of the system, leading to tight regulation in the rat brain,

this also would not have been a motivation for the average expert to replace the cassette in the adenoviral vector of Nakagawa et al. by the construct taught by Fitzsimons et al. Furthemore, nothing about the combination of these two references would give rise to a prediction or expectation of the results described above.

In view of the amendments, exhibits and arguments removal of the rejection of claims 1 and 5-7 under 35 U.S.C. §103(a) over Fitzsimons et al., supra, in view of Nakagawa et al., *European Journal of Pharmaceutical Sciences* 13:53-60 (2001) is respectfully requested.

Applicants respectfully traverse the rejection of claims 1 and 5-7 under 35 U.S.C. §103(a) over Fitzsimons et al., *supra*, in view of Lode et al., *European Journal of Pharmaceutical Sciences* 13:53-60 (2001).

<u>*Lack of Prima Facie Case*</u>

As amended, claim 1 is directed to a recombinant vector containing an insert having the general structure tTA-intron1-TK+-TetO7-CMV+-intron2-transgene, wherein the vector is an adenovirus. Fitzsimons describes a recombinant adeno-associated virus (rAAV) viral vector, but does not teach or suggest an adenoviral vector as claimed in amended claim 1. This deficiency is not cured by the secondary reference by Lode et al. To support a *prima facie* finding of obviousness, it is required that the prior art references must teach or suggest all the claim limitations. *In re Royka*, 490 F.2d 981, 985 (CCPA 1974). Applicants respectfully submit that this requirement for a *prima facie* case has not been met with regard to the instant rejection.

<u>*Unexpected results*</u>

The unexpected results achieved with the presently claimed vectors are described in detail above. The secondary reference by Lode et al. does not add anything of significance except that the authors teach single-chain IL12 fusion protein. The publication, however, is completely silent as to the cassette bearing the transgene and the combination of Fitzsimons et al. and Lode et al. provides no expectation of the results described above.

In view of the amendments, exhibits and arguments removal of the rejection of claims 1 and 5-7 under 35 U.S.C. §103(a) over Fitzsimons et al., *supra*, in view of Lode et al., *European Journal of Pharmaceutical Sciences* 13:53-60 (2001) is respectfully requested.

Regarding 35 U.S.C. §112

The rejection of claims 1-4 and 9-12 under 35 U.S.C. §112, second paragraph as allegedly indefinite is respectfully traversed.

Claim 1 is allegedly indefinite for reciting the phrase "exhibiting the general structure." Applicant maintain that, viewed in light of the specification, which discloses in paragraphs [8]-[39] of the published application, what is encompassed by the general structure. Accordingly, this phrase is submitted to be sufficiently clear and definite. Nevertheless, Applicants have amended claim 1 to replace the offending phrase with "essentially comprising."

Claim 2 is allegedly vague with regard to the term "reverse." The intended point of reference is allegedly unclear as each viral genome allegedly has various points of reference. Applicant respectfully maintain that the recited phrase itself, which states that the insert "is inserted into the viral vector genome in reverse orientation," clearly communicates to the skilled person that the viral vector genome is the point of reference such that the vector is inserted in reverse direction compared to the genome of the virus.

Claim 3 is allegedly vague because it recites the term "inverted" without a point of reference. Applicant submits that the recited phrase itself, which is "wherein the positions of tTA and transgene are inverted in the insert" makes clear that the two elements are inverted vis-à-vis each other.

Claim 4 is rejected for not describing the "use of the lac repressor." Applicants submit that claim 4 is a composition claim and there is not requirement to describe the use of lac repressor in the claim. The metes and bounds of the claim are delineated by the elements that make up the vector composition, not their use.

In view of the above, removal of the various grounds of rejecting claims 1-4 and 9-12 under 35 U.S.C. §112, second paragraph, is respectfully requested.

CONCLUSION

In light of the amendments and remarks herein, Applicants submit that the claims are now in condition for allowance and respectfully request a notice to this effect. The Examiner is invited to call the undersigned attorney if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper,

including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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RESEARCH ARTICLE

Highly suppressible expression of single-chain interleukin-12 by doxycycline following adenoviral infection with a single-vector Tet-regulatory system

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Abstract

Background Adenoviral vectors have been shown to efficiently transfer DNA into a wide variety of eukaryotic cells *in vitro* and *in vivo*. However, the therapeutic benefit of this approach is limited by severe side effects as a result of uncontrolled transgene expression.

Methods A bi-directional promoter that controls the desired transgene as well as a tetracycline-suppressible transactivator (tTA) was cloned into the E1-region of E1-deleted recombinant adenoviral vectors. Autoregulation within this construct was obtained by tTA expression under control of the operator, to which tTA binds in the absence of tetracycline. Consequently, binding of tetracycline to tTA results in downregulation of tTA as well as the co-expressed transgene in the infected cell.

Results We were able to suppress luciferase-reporter gene expression by up to 16000-fold in the presence of doxycycline (dox, 2 μg/ml). Under control of this tetracycline-regulated system, single-chain interleukin-12 (scIL12) was expressed. Adenovirally mediated expression of this potentially lethal cytokine with strong activation of antitumoral immune response was downregulated by up to 6000-fold in the presence of dox. Subsequently, this downregulation also resulted in a highly significant reduction of interferon-γ secretion by stimulated splenocytes. These mainly contribute to the toxicity of this immunotherapeutic approach.

Conclusions With expression levels exceeding those of the cytomegalovirus (CMV) promoter in almost all cell lines tested, these new vectors will also contribute to the safety of adenoviral approaches by controlled expression without compromising on maximum expression levels. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords interleukin-12; adenovirus; luciferase; tTA transactivator; Tet; doxycycline

Introduction

Adenoviral vectors provide efficient gene transfer in a wide variety of different eukaryotic cells as well as high transgene expression in cancer gene therapy. However, the therapeutic benefit of adenoviral vectors utilizing a constitutive promoter is limited by uncontrolled transgene expression and related toxicity. Our objective was to create adenoviral vectors with high transgene expression levels as well as the option to efficiently downregulate

EXHIBIT 1

the system in case of severe side effects. Expression levels in the absence of doxycycline exceed those of traditional constitutive promoters like the cytomegalovirus (CMV) promoter. This superior expression can help to reduce the viral dose administered, which will consequently limit vector-related toxicity. Transgene-related toxicity is of major concern in gene therapy approaches utilizing cytokine genes or apoptosis-inducing genes. Therefore, downregulation of transgene expression by more than three orders of magnitude will certainly contribute to the safety of these approaches if unacceptable side effects should arise.

Currently the Tet-system, which is based on elements of the *Escherichia coli* tet operon, seems to be one of the most suitable tools for the control of gene expression. The tetracycline-inducible repressor protein (tetR) is fused with the transcriptional activation domain of herpes simplex virus VP16. This tTA fusion protein interacts with the heptamerized tetO operator sequence resulting in transcriptional activation of flanking minimal promoters [1,2]. Binding of tetracycline and its derivatives to the TetR domain of tTA inhibits the interaction of this fusion protein with its operator sequences, which results in downregulation of transgene expression. Utilizing the original tet-regulated expression system in recombinant adenoviral vectors [3,4] posed two major problems. Limited packaging capacity and inappropriate activation of the CMV minimal promoter by enhancer interference are major obstacles in obtaining regulated gene expression delivered by adenoviral vectors [5]. In addition, constitutional transactivator expression led to transcriptional squelching with VP16-related toxicity. To overcome these obstacles, the tTA was expressed under the control of a bi-directional, minimal promoter consisting of the previously described heptamerized TetR binding domains flanked by minimal TK- and CMV-promoters [6].

This is the first report of the construction of adenoviral vectors delivering tetracycline-suppressible gene expression by integrating this autoregulated bi-directional expression cassette into the E1-region of a single, replication-deficient recombinant adenovirus. Expression of tTA under control of the TK minimal promoter and different transgenes under control of the CMV minimal promoter resulted in a positive feedback loop in the absence of tetracycline and its derivatives (Figure 1). The luciferase marker gene was utilized to compare adjustable transgene expression after infection of tumor cells with adenoviruses providing constitutive CMV-driven gene expression. Subsequently, this adenoviral tetracycline-suppressible gene expression system was used for the regulated expression of a

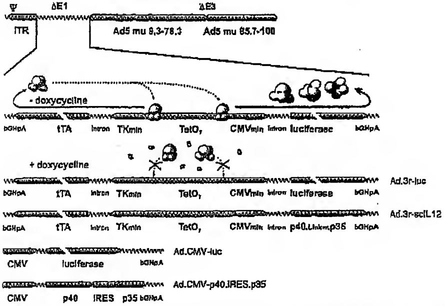

Figure 1. Autoregulated, tetracycline-dependent transactivator expression and adenoviral construction. The bi-directional tet-responsible promoter (TK_{min}-$TetO_7$-CMV_{min}) controls transgene as well as transactivator expression. Binding of the transactivator in the absence of tetracycline or doxycycline results in an amplification of transactivator expression through a positive feedback loop as well as induction of transgene expression. The autoregulated tetracycline expression cassette was inserted into the ΔE1 region of the adenoviral genome. An intron was placed upstream from the transactivator and the luciferase or murine interleukin-12 gene to prevent cryptic splicing and to obtain RNA stability (Ad.3r-luc and Ad.3r-scIL12). Moreover, recombinant adenoviral vectors for the expression of luciferase or the heterodimeric murine interleukin-12 under control of the CMV-promoter were constructed (Ad.CMV-luc and Ad.CMV-p40.IRES.p35). tTA, Tet-repressor and VP16 fusion protein; TK_{min}, thymidine kinase minimal promoter; CMV_{min}, cytomegalovirus minimal promoter; $TetO_7$, heptamerized Tet-operator. E1 and E3, Early regions of the adenoviral genome; IRES, internal ribosome entry site; CMV, cytomegalovirus promoter; TK, thymidine kinase promoter

single-chain, murine interleukin-12. ScIL12 was chosen for regulated adenoviral gene expression in cancer gene therapy because this heterodimeric cytokine efficiently stimulates antitumoral activation and proliferation of T-lymphocytes and NK-cells. Cytolytic activity of these cells is further enhanced by IL12-mediated production of Interferon-γ (IFN-γ).

IL12 has been proven to be effective in a variety of different cancer models [7,8]. IL12-secreting tumor cells were shown to induce a strong antitumoral immune response in mice. Thus, different preclinical and clinical studies using transduced tumor cells [9] or fibroblasts [10] were initiated. In order to facilitate the expression and secretion of bioactive IL12, which is normally composed of two independently transcribed subunits, a fusion protein replaced this heterodimer with a polypeptide linker in between these subunits [11,12]. Consequently, inhibition of bioactivity by the formation of p40 homodimers [13–15] was prevented.

Systemic administration of recombinant IL12 was associated with severe side effects resulting in a termination of a clinical study [16]. Considering side effects such as hemorrhagic colitis, leucopoenia, and elevated liver enzymes after systemic administration of human IL12 [17], the development of a recombinant adenoviral vector providing adjustable scIL12 expression for the transduction of tumor cells would significantly contribute to the safety of these innovative approaches in cytokine gene therapy.

Materials and methods

Cell lines

Hela, Hela.CMV-tTA, and 293 human embryonic kidney cells were cultured in HGDMEM (Gibco, Rockville, MD, USA). Human RT-4 bladder cancer cells and human colon adenocarcinoma cells HT29 were kept in McCoy medium (Gibco). MCF-7 and BT-20 human breast cancer cells as well as human colon (Colo 205 and SkCO-1) and pancreatic adenocarcinoma (Aspc-1) cell lines were grown in RPMI medium (Gibco). HepG2 human hepatocellular carcinoma cells were maintained in MEM medium (Gibco). Cells were cultured and split according to standard procedures. All media were supplemented with 10% fetal bovine serum (FBS; Tet system approved, Clontech, Palo Alto, CA, USA), 1% penicillin/streptomycin (Gibco) and 1% glutamine (Gibco). The human myeloma cell line U266 was grown in RPMI medium supplemented with 15% FBS (Clontech) and 1% penicillin/streptomycin (Gibco).

Plasmid construction

DNA fragments were separated by agarose gel electrophoresis and eluted from the agarose with the Qiagen gel extraction kit (Valencia, CA, USA). DH5alpha cells were used for plasmid propagation. Plasmid DNA was prepared using a modified alkaline lysis protocol followed by purification over a proprietary ion-exchange column according to the manufacturer's instructions (Qiagen). Before transfection LPS contaminations in plasmid DNA preparations were reduced by a Triton X-117 extraction method [18]. The plasmid pBIG 3r containing the autoregulated tTA expression system was kindly provided by C. S. Strathdee [6]. The firefly luciferase cDNA was obtained from the pGL3basic (Promega, Madison, WI, USA) by BglII and XbaI digestion and inserted into pBIG 3r cleaved with SpeI and BamHI generating pBIG 3r luc. The adenoviral plasmid pAd.CMV expression cassette was removed by XbaI and SalI digestion after refilling with T4 DNA polymerase. pBIG3r luc was digested with PvuII and SalI, and fragments containing the bicistronic expression cassette were ligated into the backbone of pAd.CMV-pA. The resulting adenoviral plasmid pAd3r-luc contained the bi-directional expression cassette 5′-flanked by the 1–456 bp of the AD5 genome including left ITR and packaging signals and 3′-flanked by 3346–5865 bp of the AD5 genome. Minimal TK-promoter-driven expression of the tTA was antiparallel, and minimal CMV-promoter-driven expression of the luciferase gene was parallel to the adenoviral E1 transcription. The luciferase gene was released from pGL3-basic by digestion with KpnI/SalI and ligated into the adenoviral expression plasmid pAd.CMV-pA resulting in pAd.CMV-luc. The scIL12 cDNA was obtained from pSFG.IL12.p40.L.p35 [11] after digestion with NcoI and EcoRV. This fragment was subcloned into the NheI/SalI site of pAd.3r-luc replacing the luciferase gene. pSFG.IL12.p40.L.p35 was kindly provided by R. C. Mulligan. The pAd.CMV.p40.IRES.p35, kindly provided by M. Caruso, contains the two murine IL12 subunits separated by an encephalomyocarditis virus internal ribosome entry site (IRES). Expression of this construct is under control of the human cytomegalovirus promoter (CMV) element from – 601 to – 14 relative to the transcriptional start site, which has been shown to efficiently drive gene expression in adenovirally transduced cells [19].

Generation and amplification of recombinant adenoviral vectors

Recombinant E1- and E3-deleted adenoviruses were rescued and plaque-purified after calcium phosphate mediated cotransfection of pAd.3r-luc, pAd.CMV-luc, pAd.3r-scIL12 or pAd.CMV.p40.IRES.p35 with pBHG10 [20]. The E1- and E3-deleted adenoviruses were amplified in 293 cells and purified by CsCl centrifugation as previously described [21]. Titration of purified viruses was performed by plaque assay. The resulting titers for Ad.3r-luc, Ad.CMV-luc, Ad.3r-scIL12 and Ad.CMV-p40.IRES.p35 were 1.0×10^{10} p.f.u./ml, 7.5×10^{9} p.f.u./ml, 6.7×10^{9} p.f.u./ml, and 8.0×10^{9} p.f.u./ml, respectively. Viral DNA was obtained (Qiagen DNA blood kit) for sequencing analysis in order to confirm insertion, transactivator sequence and orientation.

In vitro adenoviral transfection

HT29, Colo205, SkCO-1, AsPc-1, HepG2, MCF-7, BT-20 and U266 cells were seeded into six-well and twelve-well (U266) plates at a concentration of 1×10^6 cells/well 6 h before transfection. Larger Hela, Hela.CMV-tTA, RT-4 and 293 cells were seeded at a concentration of 5×10^5 cells/well. U266 myeloma cells were grown and infected in suspension culture. Purified viral particles were diluted in media without supplements, and cells were exposed to 500 µl of the appropriate virus dilution per well for 1 h. After removal of the infectious supernatant, full media supplemented with various concentrations of dox were added. Media were replaced every 24 h.

Quantification of transgene expression

Twenty-four hours post-infection with Ad.CMV-luc or Ad.3r-luc, cells were harvested with 150 µl cell culture lysis reagent according to the manufacturer's protocols (Promega). Luciferase activity in 20 µl of cell lysate was measured using a Bertold LB9507 luminometer and luciferase assay substrate (Promega). Standard curves were generated using recombinant firefly luciferase (Promega) diluted with CCLR to concentrations from 1 pg/ml to 300 ng/ml. Since rlu show a saturation profile at higher concentrations, a two-phase exponential association curve fitting was performed using the Prism software package (GraphPad Software, Inc, San Diego, CA, USA). Protein concentration was determined using the DC protein assay kit (BioRad, Hercules, CA, USA).

Quantification of single-chain and heterodimeric murine IL12 in cell-free supernatant after adenoviral infection of tumor cells was performed by a mIL12 p70 ELISA (OptEIA™, Pharmingen) assuming similar immunoreactivity and molecular weight for both forms. Splenocytes were isolated using standard procedures. Splenic cells were then cultured for 3 days with RPMI 1649 supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamine in anti-mouse CD3-coated flasks in the presence of anti-human CD28 (5 µg/ml) in order to enrich T-cells and stimulate murine IL2 secretion. Bioactivity was determined after addition of 50-fold diluted conditioned supernatant from Ad.3r-scIL12 (+/− dox), Ad.CMV-p40.IRES.p35, and mock infected HT29 cells to 4×10^4 murine splenocytes in a final volume of 125 µl for 24 h. Murine IFN-γ was quantified in splenocyte-free supernatant using a IFN-γ ELISA (OptEIA™, Pharmingen). To determine specific bioactivity, half-log dilutions of conditioned supernatant of both forms of adenovirally expressed murine IL12 and baculovirus-expressed purified murine IL12 (R&D Systems) were tested for IL12 immunoreactivity (p70 ELISA) and IFN-γ induction in splenocytes as described. Bioactivity of adenovirally expressed heterodimeric IL12 can be reduced by the formation of inhibitory p40 homodimers as described elsewhere. We did not use a capture bioassay in order to reflect potentially lower bioactivity *in vivo*.

SDS-PAGE and immunoblotting

Following infection of HT29 colon cancer cells, lysates were loaded on 15% acrylamide sodium dodecyl sulfate (SDS) gels after boiling in Laemmli sample buffers under reducing conditions. After electrophoretic separation proteins were transferred to 0.45 µm Immobilon-P (Millipore, Bedford, MA, USA) and blocked with TBS containing 5% non-fat dry milk for 1 h. Actin and the fusion protein tTA were detected using a rabbit anti-actin affinity isolated antigen-specific antibody (#A2066; Sigma, St. Louis, MI, USA) and a mouse anti-TetR monoclonal antibody (#8632-1, Clontech) [1]. After incubation for 1 h, blots were washed with TBS containing 0.1% Tween-20, pH 7.5, and incubated with anti-rabbit and anti-mouse peroxidase-linked secondary antibodies (Dianova, Hamburg, Germany) for 1 h at room temperature. Proteins were finally visualized after washing and chemiluminescence detection [22] (ECL, Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Tetracycline screening of serum from blood donors

One female and seven males aged between 23 and 35 years old were selected as volunteers. They had not received any antiinfectious treatment for at least 1 month. All of them were healthy and on a western standard diet. 50 ml of peripheral venous blood were drawn, and serum was obtained using standard procedures. Sera underwent one freeze-thaw cycle before cell culture experiments and tetracycline determinations were performed. Human sera were added to cell culture media instead of FBS. Tetracycline-HCl was purchased from Fluka Chemicals (Germany). Bakerbond RP-18 solid-phase extraction (SPE) columns were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA), HPLC-grade solvents and other chemicals were purchased from Merck (Whitehouse Station, NJ, USA). High-performance liquid chromatography (HPLC) was performed on a Constametric 3500 MS and RP-18 HyPURITY® ADVANCE columns from ThermoQuest (Germany). Data analysis was performed using Chemstation software from Agilent (Germany). After preconditioning of RP-18 columns with 2×1 ml methanol followed by 2×1 ml water, 3 ml of serum containing 0.1 mol/l citrate buffer (pH 6.8) and 0.1 mol/l EDTA were applied at a flow rate of 1 ml/min. The columns were then washed with 10 ml of water and 1 ml of methanol. Tetracycline was eluted by 4 ml of methanol containing 0.1% trifluoroacetic acid [23]. The eluate was dried and reconstituted in 100 µl 0.01% oxalic acid in water/acetonitrile (98 : 2, v/v) at pH 2.0 adjusted with HCl. Chromatography was performed at room temperature at flow rates of 0.9 ml/min. Fluorescence at 416 nm (excitation) and 515 nm (emission) was achieved by complexating tetracycline with 0.2% (w/v) zirconium(IV) chloride [24].

Calibration was performed with aqueous solutions of tetracycline-HCl at 2–100 ng/ml with coefficients of variations of 6.3% (intraday) and 8.5% (interday) for 10 ng/ml.

Results

Construction of dox-suppressible, autoregulated adenoviral vectors

The adenoviral expression plasmids pAd.3r-luc and pAd.3r-scIL12 containing the luciferase and murine scIL12 gene under control of the tetracycline-suppressible autoregulated system were constructed. In a similar procedure, plasmids containing the luciferase gene and the cDNA encoding murine p40 and p35 linked by an internal ribosome entry site (IRES) under control of the CMV-promoter were generated. Finally, recombinant E1/E3-deleted adenoviruses Ad.3r-luc, Ad.3r-scIL12, Ad.CMV-luc and Ad.CMV-p40.IRES.p35 (Figure 1) were generated by cotransfection of these adenoviral expression plasmids with pBHG10. Plaque purification and amplification were performed in 293 cells. Adenoviral titers were quantified by standard plaque assay techniques. Rescue, amplification and plaque assay of Ad.3r-scIL12 were up to 87-fold higher in the presence of dox (2 μg/ml), probably indicating the toxicity of unsuppressed scIL12 expression in 293 cells. In contrast, dox did not have an influence on the titering of Ad.3r-luc (data not shown).

Dose-dependent, doxycycline-regulated luciferase and transactivator gene expression

We have previously demonstrated the high susceptibility of human colon cancer cells HT29 to adenoviral transduction [25]. These cells were infected with Ad.3r-luc at an multiplicity of infection (MOI) of 30 following incubation with dox at various concentrations for 24 h. Luciferase activity was determined in cell lysates related to soluble cell protein. Dox concentrations as low as 100 pg/ml resulted in significant reduction of gene expression. Furthermore, gene expression was maximally suppressed with dox concentrations of up to 3 μg/ml, resulting in residual activity of 10 ng/mg (Figure 2). This concentration of dox is commonly used in the clinical treatment of bacterial infections. In this setting, dox-mediated suppression of transgene expression was up to 2400-fold. The dose-dependent, dox-regulated suppression of the positive feedback loop was illustrated by detecting the tTA fusion proteins with TetR monoclonal [1] and VP16 polyclonal antibodies [26] in Western blot analysis. Increasing concentrations of dox resulted in downregulation of intracellular tTA levels, correlating with decreased luciferase gene expression.

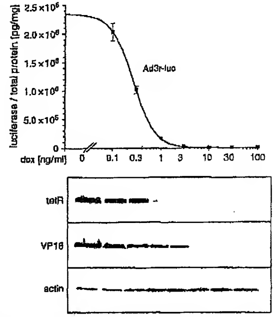

Figure 2. Dose-dependent gene expression after infection of HT29 colon cancer cells with Ad.3r-luc followed by various concentrations of the tetracycline derivative doxycycline. Luciferase expression was suppressible by doxycycline at concentrations as low as 0.1 ng/ml. Western blot analysis of the transactivator revealed the positive feedback loop with suppression of tTA fusion protein expression in the presence of doxycycline. Dox, doxycycline; tetR, Tet-repressor; VP16, herpes simplex virus transcriptional activation domain

MOI-dependent, suppressible luciferase expression

HT29 cells were infected with Ad.3r-luc at MOIs from 0.1 to 100 following incubation in the absence or presence of dox at 2 μg/ml for 24 h. Suppression of luciferase gene expression in lysates of Ad.3r-luc-infected HT29 cells ranged from 470 (MOI 0.3) to 2400-fold (MOI 10–100) (Figure 3). The extent of suppression remained constant at high MOI, which could be crucial for sufficient control over transgene-related toxicity. Dox at concentrations of 2 μg/ml did not impair adenoviral gene expression in HT29 cells utilizing the constitutive CMV-promoter (data not shown). In order to investigate the efficiency of Ad.3r-luc-mediated transgene expression in the absence of dox, expression was compared with HT29 cells infected with Ad.CMV-luc. In HT29, Ad.3r-luc revealed higher gene expression than Ad.CMV-luc for all MOIs tested (1–100) ranging between 18-fold (MOI 100) and 240-fold (MOI 1).

MOI-dependent regulated expression of murine interleukin-12

HT29 cells were infected with Ad.3r-scIL12 at MOIs ranging from 1–100 and incubated in the absence

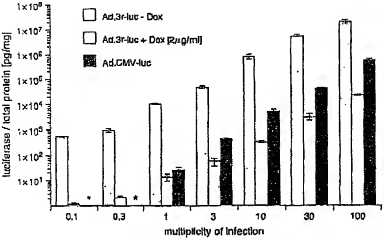

Figure 3. Luciferase gene expression after infection of HT 29 cells with Ad.CMV-luc or Ad.3r-luc at various multiplicities of infection (MOI). Doxycycline-regulated gene expression after infection with Ad.3r-luc was achieved within a wide range of infection from at least 0.1 to 100 MOI, resulting in 470- to 2400-fold suppression of luciferase expression. Luc expression was considerably lower using the CMV-promoter instead of the 3r-promoter system in the absence of dox at all MOI tested

or presence of dox at 2µg/ml for 24 h. ScIL12 gene expression was suppressed by more than 1400-fold at an MOI of 100 in the presence of dox (Figure 4). Western blot analysis revealed transactivator (tTA) expression correlating with IL12 expression. Comparison with CMV-driven IL12 expression confirmed previous findings using luciferase-expressing adenoviruses. Nonsuppressed 3r-mediated IL12 gene expression was 11-fold (MOI 1) to 375-fold (MOI 100) higher than utilizing the constitutive CMV-promoter, assuming similar immunoreactivity of the p70 ELISA towards scIL12 and the CMV-driven expression of a p40/p35 heterodimer. Bioactivity of both forms was quantified by incubation of murine splenocytes with 50-fold diluted conditioned media following infection of HT29 with IL12-expressing adenoviruses (Figure 5). High IFN-γ secretion of splenocytes was obtained by incubation with conditioned media after infection of HT29 with Ad.3r-scIL12. This induction of IFN-γ was significantly higher as compared with infection of HT29 with the commonly used Ad.CMV-p40.IRES.p35. Addition of dox resulted in a suppression of IFN-γ to background levels. Furthermore, the specific bioactivity of adenovirally expressed forms of IL12 in comparison with recombinant purified p40/p35 heterodimers was analyzed (Figure 6). Murine splenocytes were incubated with half-log dilutions of recombinant heterodimeric IL12 or IL12 containing conditioned media as described. Induction of IFN-γ correlated with immunoreactivity of IL12 in the media as determined by p70 ELISA. Basal induction was caused by preincubation of splenocytes with anti-human CD28 antibodies resulting in the expression of IL2 and subsequent induction of IFN-γ [27]. Bioactivity of the murine single-chain IL12 fusion protein was comparable to the purified recombinant p40/p35 heterodimer. The reduced bioactivity of IL12 expressed after infection with the commonly used Ad.CMV-p40.IRES.p35 could be explained by inhibitory p40 homodimers as previously described [13–15].

Regulated luciferase and interleukin-12 gene expression *in vitro*

Different human colon carcinoma (HT29, SkCo-1 and Colo205), pancreatic carcinoma (Aspc-1), bladder carcinoma (RT4), cervical carcinoma (HeLa), breast carcinoma (MCF-7 and BT-20) as well as myeloma (U266) and hepatocellular carcinoma (HepG2) cell lines were infected with either Ad.CMV-luc, Ad.3r-luc, Ad.CMV-p40.IRES.p35 or Ad.3r-scIL12 and incubated in the presence or absence of dox. Expression of luciferase and recombinant interleukin-12 was determined as previously described. Dox-mediated suppression of luciferase and interleukin-12 expression was illustrated in all cell lines (Figure 7). Dox resulted in a 4.4-fold (U266) to 16 000-fold (SkCo-1) suppression of luciferase expression. Suppression of IL12 was 3.9-fold in U266 and ranged from 167-fold (HepG2) to 6000-fold (Aspc-1). In the absence of dox the 3r-promoter proved to be superior to the CMV-promoter in almost all cell lines tested. Except in HepG2 with high CMV-mediated luciferase expression which is comparable to 3r-mediated expression, in all other cancer cell lines the 3r-promoter resulted in 4.1-fold (U266) to 1100-fold (HT-29) higher gene expression in the absence of dox. Similar differences were found between CMV-mediated and 3r-mediated interleukin-12 expression. Interleukin-12 expression in mock-transfected cell lines was not detected. Except in U266 with significantly lower 3r-mediated IL12 expression, the 3r-promoter resulted in 17-fold (SkCo-1) to 4254-fold (Colo205) higher transgene expression.

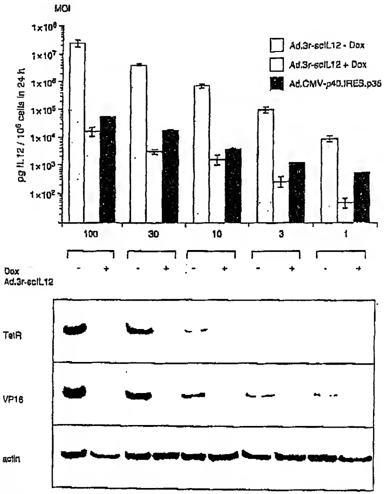

Figure 4. Interleukin-12 and tTA expression after infection of HT29 with interleukin-12 expressing adenoviruses at various MOI. As shown with luciferase-expressing adenoviral vectors, in HT29 there was a substantially higher yield of interleukin-12 expression when the 3r-promoter was used instead of the CMV-promoter. Addition of doxycycline resulted in suppression of transgene expression even below levels obtained by Ad.CMV-p40.IRES.p35 at same MOI. Both domains of the tTA fusion protein were detected using TetT and VP16 antibodies after infection with Ad.3r-scIL12. Expression of the tTA fusion protein correlated with the MOI used. Addition of doxycycline at 2 µg/ml resulted in suppression of tTA expression. TetR, tetracycline-repressor; VP16, herpes simplex virus transcriptional activation domain

Luciferase gene expression after infection of tTA-transformed Hela cells with Ad.3r-luc

Hela cells with stable transformation of CMV-driven tTA expression were compared with native Hela cells after infection with Ad.3r-luc in the presence and absence of doxycycline in order to determine effects of autoregulated tTA expression in comparison to constitutively high intracellular levels of tTA (Figure 8). Excess of tTA in transformed Hela resulted in a 310-fold increase of CMV_{min}-mediated gene expression at an MOI of 1. This difference became less than 5-fold (MOI 100) which was due to high intracellular tTA levels in normal Hela cells at high MOI. Dox-mediated suppression of gene expression was seen in both cell lines at all values of MOI with 2.5-fold higher gene expression in CMV-tTA-transformed cells at all MOI tested. Higher leakiness in the presence of dox indicated residual activation of the CMV_{min}-promoter by constitutive transactivator expression in Hela.CMV-tTA. Leakiness was reduced by autoregulated tTA expression.

Regulated gene expression following incubation with human serum

Only very low concentrations of doxycycline and tetracycline (tet) are necessary to suppress transgene expression in our new system. Therefore, regulation

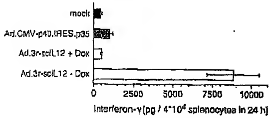

Figure 5. Induction of interferon-γ expression after incubation of splenocytes with conditioned supernatant of infected HT29 cells. 10^6 HT29 cells were infected with Ad.3r-scIL12 (+/− Dox) or Ad.CMV-p40.IRES.p35 at an MOI of 30 for 24 h. Infection of HT29 with Ad.3r-scIL12 resulted in high levels of interferon-γ induction as compared with infection with Ad.CMV-p40.IRES.p35. Addition of doxycycline resulted in a reduction of interferon-γ to background levels in this bioassay

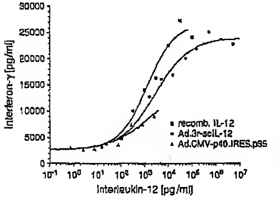

Figure 6. Comparison of interferon-γ induction by adenovirally expressed single-chain and heterodimeric interleukin-12 as well as purified recombinant interleukin-12. Interleukin-12 was determined in conditioned supernatant of infected HT29 cells utilizing a p70-mIL12 ELISA. Murine splenocytes were then incubated with serial dilutions of either adenovirally expressed or recombinant interleukins, and induced interferon-γ was quantified using a mIFN-γ ELISA. The bioactivity-immunoreactivity of single-chain interleukin-12 was comparable to recombinant purified heterodimeric interleukin-12. Specific bioactivity of adenovirally produced heterodimeric interleukin-12 (Ad.CMV-p40.IRES.p35) appeared to be lower, possibly due to inhibitory p40 homodimers

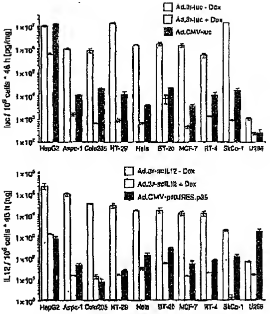

Figure 7. Luciferase and interleukin-12 expression after infection of different cell lines with either CMV-driven or 3r-driven gene expression in the presence and absence of doxycycline (MOI 10; U266: MOI 200). Intracellular luciferase gene expression was superior to the 3r-promoter in all cell lines tested. Addition of doxycycline resulted in highly significant suppression of gene expression, which was even lower than CMV-promoter-driven gene expression. Interleukin-12 levels in the supernatant after infection with the corresponding IL12-expressing adenoviruses revealed a similar pattern except for the U266 cell line

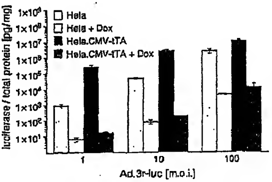

Figure 8. Luciferase gene expression after infection of Hela and Hela-CMV-tTA cells with Ad.3r-luc. In the presence of dox (2 μg/ml) there was only a minimal difference in transgene expression (Hela + Dox vs. Hela.CMV-tTA + Dox). In dox-free medium, infection of stable tTA-transformed Hela cells resulted in more than 2-log higher luciferase gene expression at an MOI of 1 with decreasing differences between these cell lines at higher MOI (Hela vs. Hela.CMV-tTA)

of gene expression in human colon cancer cells was investigated in the presence of human serum to confirm the feasibility of this approach in a possible clinical setting. Since tetracycline is widely used in stock breeding, serum samples from healthy volunteers on a standard western diet were tested for tet using a standard HPLC procedure with a maximum sensitivity of 2 ng/ml. HPLC did not reveal significant tet concentrations in any of the samples tested. HT29 colon carcinoma cells infected with Ad.3r-luc (MOI 30) following incubation with these human sera did not result in a significant difference in transgene expression as compared with certified tetracycline-free fetal bovine serum (Figure 9). This finding reflects tetracycline concentrations in human serum samples less than 10 pg/ml. As expected, supplementation of these human sera with dox (2μg/ml) resulted in highly efficient suppression of transgene expression. Interestingly, this

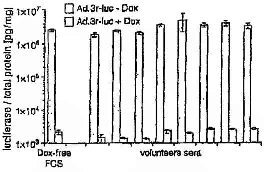

Figure 9. Incubation of Ad.3r-luc-infected HT 29 colon carcinoma cells with human sera instead of certified tetracycline-free fetal bovine serum. There was no significant difference using human serum of volunteers on a standard western diet as compared with certified tetracycline-free fetal calf serum. These data suggest tetracycline concentrations of human volunteers to be below 50 pg/ml. Supplementation of human sera with doxycycline (2 μg/ml) resulted in suppression of transgene expression as previously shown. FCS, fetal calf serum

biological system is at least 40-fold more sensitive than standard HPLC procedures implicating a more suitable tool for the detection of tet and dox traces in human and veterinary medicine [28].

Discussion

With the development of potent viral vector systems enabling efficient gene transfer *in vitro* and *in vivo*, toxicity of expressed transgenes after the event of infection is becoming one of the major concerns in cancer gene therapy. Previous reports indicated dose-dependent antitumoral cellular immune response after transduction of tumor cells with interleukin-12 [29,30]. Preclinical *in vivo* studies suggest that the therapeutic window of interleukin-12 is rather small and depends on the tumor model [11,12], genetic heterogeneity [31] and treatment schedule [16]. Thus, tight regulation of transgene expression without compromising on maximally attainable levels of gene expression through adenoviral vectors would be desirable. Dox-dependent induction of gene expression after infection with two adenoviral vectors was first described in 1998 [32]. More recent publications of tetracycline-regulated expression systems in adenoviral vectors lacking autoregulatory elements indicate significantly lower levels of regulation [33]. With two adenoviral vectors, 300-fold suppression *in vitro* and 10-fold suppression *in vivo* were obtained by a tetracycline-regulated vector system. One of the vectors contained the CMV-driven tTA gene and the other the IL12 gene under control of a tetracycline-responsive element [34]. Similar suppression was shown in a rat model [35].

We have generated and characterized a one-vector based, replication-deficient adenoviral system with autoregulated transactivator expression that allows for very tight control of transgene expression by the addition of doxycycline at non-toxic concentrations. High suppression of gene expression was accomplished over a wide range of MOI and in different carcinoma cell lines. The level of suppression depends on the concentration of dox. Maximum suppression of transgene expression was achieved within concentrations as low as 2 μg/ml, thus suitable for the clinical environment. Levels of reporter gene expression using this regulatory system exceeded levels obtained by the widely used constitutive human cytomegalovirus (HCMV) immediate-early promoter by up to more than 1000-fold in a variety of cancer cell lines in the absence of dox. Using these innovative autoregulated recombinant adenoviral vectors, the presence of transactivators prior to infection is not required. In addition, toxicity due to constitutive expression of the transactivator and transcriptional interference were avoided by dox-dependent autoregulative gene expression. These adenoviral vectors prove to be much more versatile and effortless tools, compared with transgenic animal models and cell lines that constitutively express the transactivator [1,36–38]. Dox-regulated gene expression can also be carried out after adenoviral infection of a wide variety of native mammalian cell lines or tissues. Furthermore, the autoregulation described restricts leakiness of transgene expression by reduced transactivator expression when suppressed by dox [39]. The use of a genetically engineered and highly secretory IL12 adds to the superior promoter activity resulting in up to 4000-fold higher cytokine secretion (Colo 205) compared with the HCMV-promoter-driven heterodimer in a variety of cancer cell lines. IL12 expression in the absence of dox was also superior to previous findings in which murine tumor cells were adenovirally transduced using the CMV-promoter to drive expression of the heterodimeric or scIL12 (7490 ng/10^6 cells × 48 h [11], 60 μg/10^6 cells × 120 h [40] and 1.2–5.6 ng/10^6 cells × 24 h [12]). Reported transduction of human tumor cells in preclinical (25–50 ng/10^6 cells × 24 h [41]) or clinical trials (0.987 ng/10^6 cells × 24 h [10]) was significantly lower. ScIL12 was shown to have a similar bioactivity as compared with the widely used heterodimeric form [11]. Furthermore, inhibition of bioactivity by formation of p40 homodimers was avoided and fast regulation of gene expression in the 3r-system ensured efficient secretion of scIL12.

Initially, 3r- and CMV-promoter activities were analyzed in a variety of cell lines by expression and quantification of intracellular luciferase without considering different glycosylation capacities for the secretion of IL12 (Figure 7). The 3r-promoter system was superior in all cell lines except in HepG2, which had high adenoviral transduction efficiency resulting in similar expression and only 10-fold dox-mediated suppression. In contrast, expression of secretory cytokines like IL12 resulted in far superior levels and suppression performance in the supernatant of infected cells when utilizing the 3r-promoter (Figure 7). As a result, secretory capacities of the expressed cytokines are likely to enhance the performance of these adenoviral

vectors. Secretion of IL12 depends on posttranslational processing. Unlike scIL12, which is secreted by a secretory leader domain of p40, secretion of heterodimeric IL12 is mediated by glycosylation of the p35 subunit [42,43]. Different pathways of secretion and glycosylation capacities might explain differences in transgene expression by U266. The *h*CMV-promoter we used provided IL12 expression levels in U266 cells similar to previous findings [41]. However, further studies are necessary to fully explore this phenomenon of transgene-dependent promoter performance.

The superiority of the 3r-promoter illustrates the possibility of reducing the adenoviral dose of these new adenoviral vectors, thus diminishing vector-specific side effects. This advantage is mainly attributed to VP16-mediated transactivation of transgene expression and positive feedback of tTA expression in the absence of dox. To a minor extent, differences in 3r- and CMV-promoter activity may also be attributed to differences between expression cassettes. A chimeric intron derived from the fused tripartite leader sequence of adenovirus major late transcript and a human IgG gene were integrated upstream of the tTA coding region [6,44]. These vectors were compared with widely used recombinant adenoviral vectors with CMV-promoter-driven gene expression that was not further enhanced by the integration of introns [19,25,45,46]. Enhancement of CMV-driven gene expression by intron integration has been discussed previously [47,48]. Also, downregulation of CMV-mediated gene expression by cytokines like IFN-γ or IL10 [49] needs to be considered in the clinical setting.

In order to address the question of sufficient tTA expression after adenoviral transduction with this system, we infected Hela and Hela.CMV-tTA cells with Ad.3r-luc at different MOI in the presence and absence of dox (Figure 8). In the presence of dox, suppressed gene expression was only moderately higher in Hela.CMV-tTA at all values of MOI tested. Without dox, infection of constitutively tTA-expressing HeLa resulted in more than 300-fold higher transgene expression at low MOI, but only 5-fold higher gene expression at high MOI. Adenovirally expressed tTA quantitatively compared with constitutively expressed tTA only at an MOI of 100, thus resulting in similar transgene activation. At low MOI, maximum transgene expression might be improved by higher tTA expression without compromising on leakiness. Nuclear targeting was utilized by incorporating the adenovirus E1A nuclear localization signal to improve tTA function in our constructs [2,6]. As shown previously [6], maximum tTA expression can be further improved by replacing the TK_{min}- with the CMV_{min}-promoter, but basal level transcription will be considerably higher compromising the performance of dox-mediated suppression.

High constitutive expression of tTA not only results in VP16-mediated cellular toxicity [50,51], but also contributes to the leakiness of tet-regulatory gene expression systems. As shown earlier, adenovirally mediated transgene expression depends on site-specific enhancer effects [33]. Read through activity by 5′-flanking E1A enhancer elements can significantly contribute to transgene expression of genes integrated into the left part of the E1 region [52]. Low background tTA expression levels in our constructs were accomplished by inserting the tTA gene in reverse orientation into the center of the E1 region. This might also explain low leakiness as compared with previously published adenoviral constructs utilizing constitutive transactivator gene expression with tetracycline-responsive elements (TRE) in the original orientation and 5′-flanked by adenoviral sequences [3–5].

Potential contamination of food with small amounts of tetracycline and its derivatives was a major concern in the use of these vectors and their sensitive autoregulated system in a clinical setting. According to our findings, a standard western diet does not seem to affect the sensitive tet-OFF system (Figure 9), although these data have to be validated in a larger study. Tet levels were lower than 50 pg/ml, thus not affecting the tet-OFF system in a potential clinical study.

One promising approach is to combine this highly efficient dox-mediated gene expression system with tissue or tumor specificity. Currently, we are investigating different modifications of the TetO-flanking minimal promoters in order to reduce transgene expression in adjacent nonmalignant tissue even in the absence of dox.

To our knowledge, our study is the first to describe and characterize single adenoviral vectors with integrated autoregulatory transactivator expression resulting in very high transgene expression in the absence of dox. Nevertheless, suppression of transgene expression by addition of this antibiotic was not impaired, and suppression levels of up to 16 000-fold were obtained. These findings illustrate the potential for the construction of regulated adenoviral vectors expressing therapeutic transgenes of up to 4.8 kb and we are confident that these adenoviral vectors will become powerful tools in the molecular therapy of malignant diseases.

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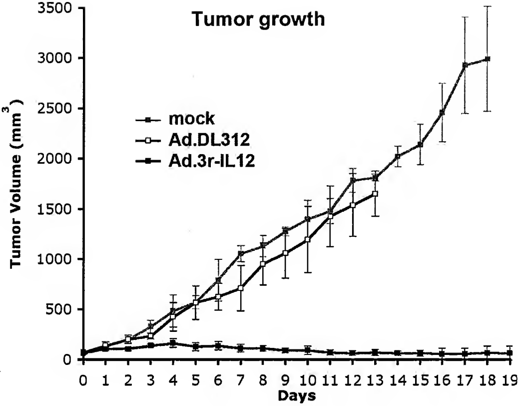

Fig. 2 Tumor progression of MCA-26 tumors following intratumoral injection of 10^9 p.f.u./20g KG Ad.DL312 or Ad.3r-IL12 10^9 p.f.u./20g KG

EXHIBIT 2

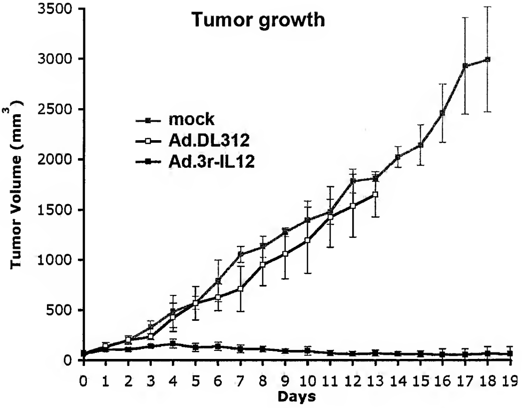

Fig. 2 Tumor progression of MCA-26 tumors following intratumoral injection of 10^9 p.f.u./20g KG Ad.DL312 or Ad.3r-IL12 10^9 p.f.u./20g KG

EXHIBIT 3

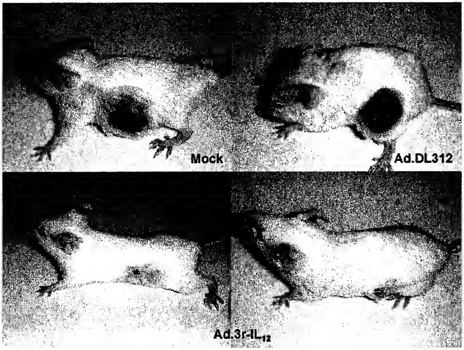

Fig. 3 Tumors at day 18. Tumor regression following intratumorale injection of Ad.DL312 (control vector) and Ad.3r-IL12 as compared to control (mock).

EXHIBIT 4